NF-κB is activated and promotes cell death in focal cerebral ischemia

ARMIN SCHNEIDER[1,2], ANA MARTIN-VILLALBA[1,3], FALK WEIH[4], JOHANNES VOGEL[5], THOMAS WIRTH[6] & MARKUS SCHWANINGER[1]

[1]Department of Neurology, University of Heidelberg, Im Neuenheimer Feld 400, [2]Department of Neuroscience, BASF-LYNX Bioscience AG, Im Neuenheimer Feld 515, and [3]Department of Physiology, Im Neuenheimer Feld 326, 69120 Heidelberg, Germany, [4]Forschungszentrum Karlsruhe, Institute of Genetics, PO Box 3640, 76021 Karlsruhe, Germany and [5]Institute of Radiobiology and Cell Research, Versbacherstr. 5, 97078 Würzburg, Germany

Correspondence should be addressed to M.S.

The transcription factor NF-κB is a regulator of cell death or survival. To investigate the role of NF-κB in neuronal cell death, we studied its activation in a rodent model of stroke. In the ischemic hemisphere, NF-κB was activated, as determined by increased expression of an NF-κB-driven reporter transgene, nuclear translocation of NF-κB in neurons and enhanced DNA binding of NF-κB subunits RelA and p50. In p50 knockout mice, ischemic damage was significantly reduced. This indicates a cell death-promoting role of NF-κB in focal ischemia. NF-κB may provide a new pharmacological target in neurologic disease.

Stroke is the third most common cause of death and the chief cause of disability in the United States and Europe. After years of setbacks, treatments for acute stroke have finally emerged, including aspirin[1]. However, for most patients, a satisfactory therapy is still lacking. The outcome and infarction size after focal cerebral ischemia is determined in part by delayed neuronal cell death in the borderzone of ischemia. This process is mediated by programmed cell death[2,3], which can be influenced in animal models to minimize damage after cerebral infarction[4].

Although originally discovered as an important transcription factor in the immune system, NF-κB has since been shown to be a primary regulator of programmed cell death in a variety of experimental settings. The function of NF-κB in cell death seems to be very dependent on the system and type of stimulus examined, and can be pro- or anti-apoptotic[5].

NF-κB is expressed in many cell types in the nervous system[6] and is constitutively active in subsets of cells in the cortex and hippocampus of the rodent brain at comparably low levels[7,8]. In addition, inducible NF-κB-activity in neurons has been found in experimental situations such as glutamate stimulation or seizure activity[9,10]. However, the functional significance of these findings is still unclear.

NF-κB is activated by a variety of stimuli known to occur in focal ischemia, such as glutamate[11], TNFα, hypoxia, reactive oxygen species and IL-1α (ref. 12). Furthermore, it is a transcriptional activator of many genes involved in the pathogenesis of cerebral ischemia (for example, iNOS, IL-1α, TNFα, ICAM1, cox-2 and IL-6; ref. 12). NF-κB-activity consists of five known subunits in mammalian cells, p50, p52, RelA, RelB and cRel, that can form homo- and heterodimers. In unstimulated cells, NF-κB complexes remain in the cytoplasm through association with inhibitory IκB proteins that mask their nuclear localization signal. After activating signals, IκB is phosphorylated and proteolytically degraded, resulting in NF-κB translocation to the nucleus[13]. NF-κB does not require *de novo* synthesis, and thus would be ideally suited to regulate gene transcription in the pathophysiology of focal cerebral ischemia, integrating extracellular stimuli to a concerted activation of target genes.

In vivo induction of NF-κB in cerebral ischemia

To determine whether focal cerebral ischemia leads to a change in NF-κB activity, we used the transgenic mouse line κB5. This mouse has a chimeric β-globin gene under exclusive transcriptional control of NF-κB consensus binding sites[14]. We induced transient focal ischemia (2-hour ischemia and 20-hour reperfusion) using the filament model. Quantitative RT-PCR (ref. 15) consistently showed 300% increased expression of the β-globin reporter gene in the ischemic hemisphere, compared with that of the non-ischemic hemisphere (317 ± 33%; $n = 3$; Fig. 1).

To obtain information on the distribution of the NF-κB activation observed, we used an antibody raised against the nuclear localization signal of the NF-κB subunit RelA (ref. 16). This epitope is not accessible in the inactive form of NF-κB in the cytoplasm, where it is masked by IκB proteins. Because of background problems in the mouse with this monoclonal antibody, we used ischemic tissue from rats operated on according to the same protocol. Middle cerebral artery occlusion results in brain damage in both mice and rats[17,18]. After 2 hours of middle cerebral artery occlusion and 20 hours of reperfusion, nuclear translocation became evident in the striatum (Fig. 2*a*) and was enhanced after 72 hours of reperfusion in the penumbral cortex, anterior thalamus and hippocampal CA-1 region (Fig. 2*b–d*).

According to morphological criteria, the positive cells seemed to be neurons. Double-fluorescence immunohistochemistry with the astrocyte marker GFAP and an antibody specific for activated RelA excluded astrocytes as the main source of NF-κB activation in our model (data not shown). Furthermore, double labeling with antibodies against the neuronal marker neurofilament-200 kD and RelA indicated co-localization (Fig. 3*a*). Most dying cells in our model were neurons, as demonstrated by TUNEL labeling and counterstaining with the neuron-specific NeuN antibody (Fig. 3*b*).

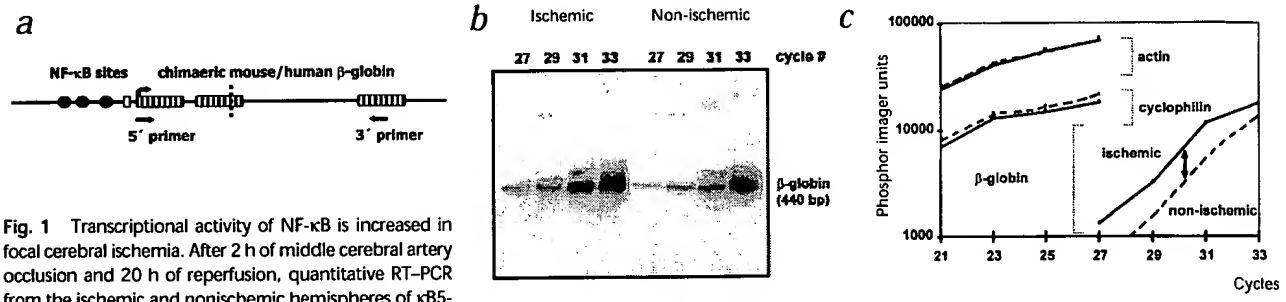

Fig. 1 Transcriptional activity of NF-κB is increased in focal cerebral ischemia. After 2 h of middle cerebral artery occlusion and 20 h of reperfusion, quantitative RT–PCR from the ischemic and nonischemic hemispheres of κB5-transgenic mice[14] was done for the κB-dependent β-globin reporter gene (*a* and *b*) and the 'housekeeping' genes β-actin and cyclophilin (not shown). *a*, Striped boxes, exons; octagons, NF-κB binding sites; shaded box, TATA box. Gel-separated PCR products were quantified with a phosphorimager (cycle numbers above blot and below graph). Ratios of β-globin:β-actin/cyclophilin for each hemisphere were calculated from the linear range of the PCR (*c*).

NF-κB activity in cerebral ischemia consists of p50 and RelA

Activation of NF-κB is due to increased DNA binding of NF-κB after its release from IκB. To determine which subunits mediate the observed upregulation of NF-κB activity, we assessed DNA binding in brain extracts with gel-shift assays. Two bands could be detected in mouse brain (Fig. 4*a*). These bands are specific for NF-κB, as they could be completely abolished by competition with the κB oligonucleotide itself, but not with a mutated binding motif (Fig. 4*a*). Complex I contained RelA/p50 heterodimers, whereas complex II consisted of p50 homodimers, as demonstrated by experiments with specific antisera against various NF-κB subunits (Fig. 4*b*).

After 2 hours of middle cerebral artery occlusion and various periods of reperfusion, there was an increase in specific NF-κB binding activity of both complexes, with an apparently higher increase in the RelA/p50 heterodimer (complex I) (Fig. 4*a*). No additional specific bands appeared in extracts from ischemic hemispheres. Gel-shift assays at different reperfusion times (30 minutes, 20 hours, 72 hours) from several mice (n = 15) showed similar results (data not shown). These data show that the increased transcriptional NF-κB activity observed is due to an induction of DNA binding of p50 and RelA.

Infarct volumes are different in p50$^{-/-}$ mice

To determine the functional importance of NF-κB activation in cerebral ischemia, we used mice deficient in the p50 subunit of NF-κB. Mice with a targeted disruption of this subunit are viable, show no developmental abnormalities, and only demonstrate specific defects in immune responses[19]. Thus, these mice are suitable to assess the importance of the observed NF-κB upregulation, as p50 is a constituent of both complexes found in brain. To verify the absence of NF-κB activity from brain in these mice, we did gel-shift assays with extracts from an ischemic knockout mouse (2-hour ischemia and 30-minute reperfusion). Both bands seen in brain extracts from wild-type mice were absent, and no new complexes were detectable (Fig. 4*c*).

The p50 knockout mice and control mice were matched for age (mean, 110 days), sex and weight (mean, 23 g). The following physiological parameters were controlled and did not differ substantially between p50 knockout mice and control mice: blood pressure, blood pH, blood glucose, pO_2 and pCO_2 (data not shown). Mean cerebral perfusion measured by laser Doppler flowmetry at the time of middle cerebral artery occlusion decreased to 22.52 ± 5.47 % in wild-type mice and 20.51 ± 6.90 % in p50 knockout mice, which was not substantially different.

The mean infarct volume after 2 hours of middle cerebral artery occlusion and 20 hours of reperfusion in control mice

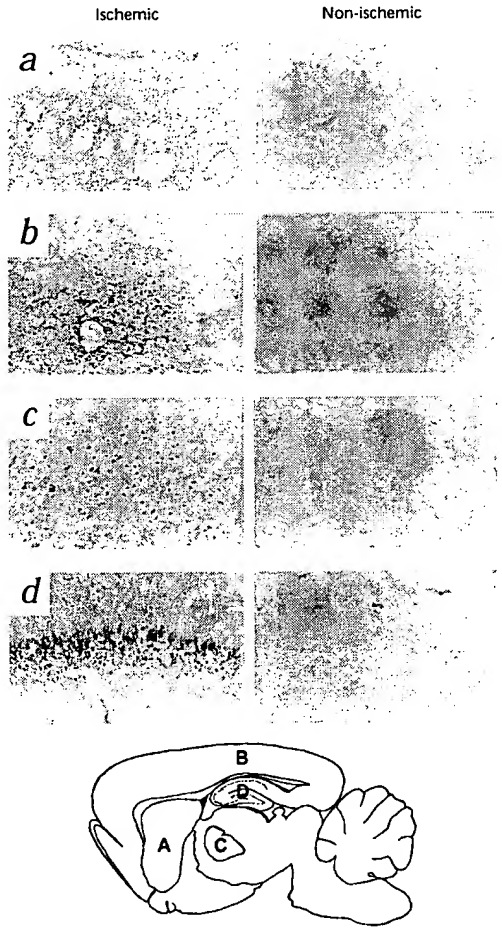

Fig. 2 Increase of NF-κB activity is confined to distinct brain areas. Left, immunohistochemistry on rat brains after focal ischemia using a RelA antibody directed against the nuclear localization signal of RelA, which is inaccessible in the inactive state. After 2 h of media occlusion and 20 h of reperfusion, reactivity was detectable in the striatum (*a*), whereas after 72 h of reperfusion, staining became obvious in the penumbral area in the cortex (*b*), the anterior thalamus (*c*) and the hippocampus CA1 region (*d*). Bottom, approximate positions of sections in *a*–*d*. Right, corresponding regions of the non-ischemic hemisphere of the same animal.

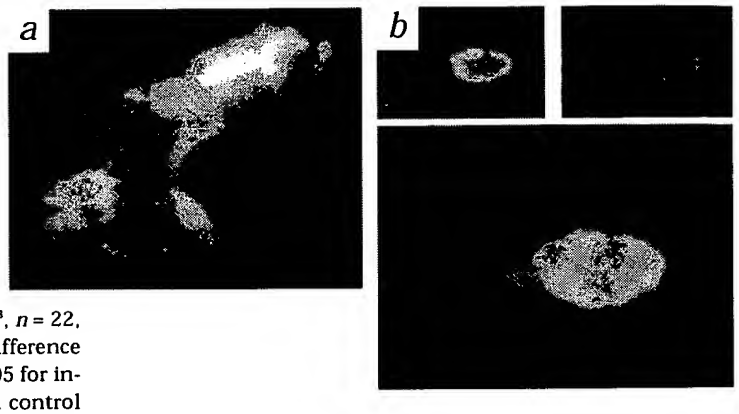

Fig. 3 Activated NF-κB localizes to neurons. Double fluorescence immunohistochemistry on the striatum of ischemic rat brains. *a*, Antibodies against activated RelA (Cy-3, red) and neurofilament-200 kD (fluorescein, green) demonstrate co-localization. *b*, Antibody against the neuronal nuclear marker NeuN (Cy-3, red) and TUNEL labeling (fluorescein, green) demonstrate a neuronal nucleus undergoing chromatin condensation at the nuclear membrane.

was in good agreement with similar models from other groups[17]. The infarct volume differed between control mice and p50 knockout mice (42.26 ± 7.69 mm^3, $n = 22$, and 32.97 ± 10.15 mm^3, $n = 15$, respectively). This difference was significant in the mid-portion of the infarct ($P < 0.05$ for infarct volume over sections 13–16: 14.20 ± 1.21 mm^3 in control mice, compared with 8.78 ± 2.45 mm^3 in p50 knockout mice; Fig. 5*a*). The sectional area distribution of the right brain did not differ between p50 knockout and control mice (Fig. 5*b*). Our study focuses on the first 22 hours after injury. Changes due to delayed neuronal death may be more profound after longer time periods, but this remains to be investigated.

We analyzed the regional infarct distribution in the coronal plane by averaging infarct areas (Fig. 5*a*, section 14). The resulting frequency density map indicates a relative sparing of the area corresponding to the hippocampus and the upper thalamus in p50 knockout mice (Fig. 5*c*).

Excitotoxicity in neuronal cell culture models some aspects of stroke[20]. Differentiated human hNT cells possess many features of mature neurons and are vulnerable to glutamate[21]. We verified the effect of glutamate (1 mM) on hNT cells by counting dying cells (47.7 ± 3.7 %) identified after a nuclear stain with Hoe 33342. This number was similar to published results[21]. We next used an ELISA assay that detects DNA/histone complexes, to measure apoptosis in relative units. To inhibit NF-κB activity, we treated hNT cells with an oligonucleotide 'decoy' containing a consensus NF-κB binding site. At 24 hours after exposure to a glutamate pulse (1 mM), we assayed a cell extract for the presence of DNA–histone complexes. Glutamate in the presence of the mutated NF-κB binding site resulted in an induction of apoptosis (100.0 ± 8.8 relative units with glutamate compared with 20.3 relative units without glutamate) that was almost halved by the consensus motif (53.3 ± 15.1 relative units with glutamate compared with 17.9 relative units without glutamate; $P < 0.05$; $n = 3$).

Discussion

Here we have shown activation of NF-κB in focal cerebral ischemia, a model of human stroke. In accordance with the present understanding of NF-κB, its activation in cerebral ischemia is a multistep process. It involves nuclear translocation of the subunits RelA and p50, increased DNA binding of p50 homodimers and p50/RelA heterodimers, and induction of transcriptional activity. NF-κB-directed gene transcription is elevated 300% in the ischemic hemisphere, as determined by marker gene expression in the κB5 transgenic mouse. However, the degree of induction in individual cells is probably higher, as immunohistochemical findings indicate a confined activation in a subset of neurons. This view is supported by data on global cerebral ischemia, where activation in hippocampal neurons has

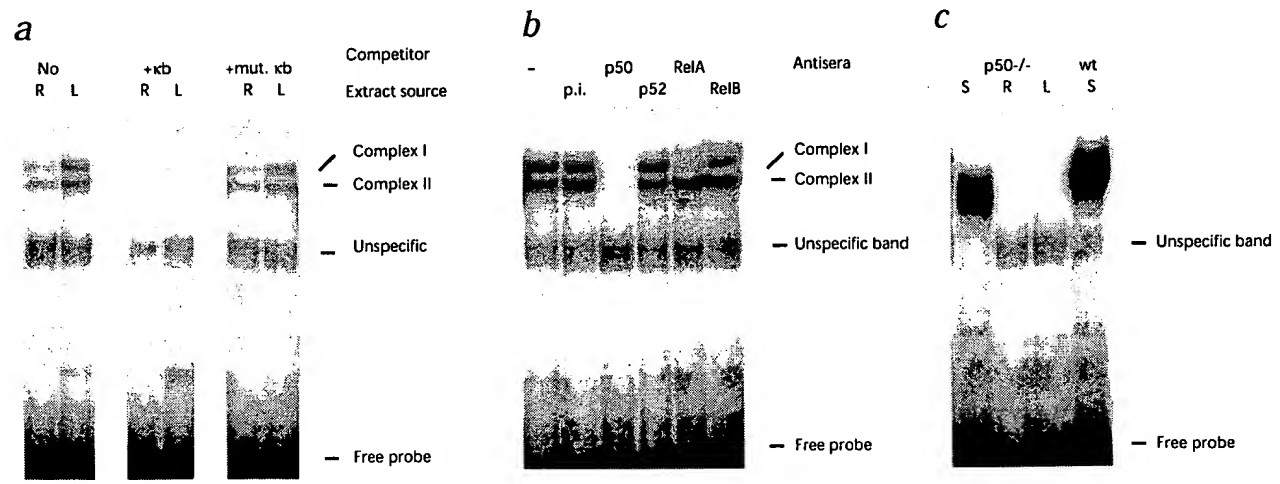

Fig. 4 Increased DNA binding of p50 and RelA in cerebral ischemia. Ischemic hemispheres (L) and non-ischemic hemispheres (R) of mice subjected to 2 h of media occlusion and 30 min of reperfusion. *a*, Two specific complexes are enhanced by ischemia. Competition with 100-fold excess of the κb-oligonucleotide itself or a mutated motif, mut.κb., demonstrates specificity of binding. *b*, Antiserum against p50 abolishes binding of complex I and II, whereas antiserum against RelA diminishes binding of complex I only. Pre-immune serum (p.i.) and antisera against p52 or RelB had no effect. –, no antiserum. *c*, Protein extracts (40 μg each) from p50 knockout mice subjected to focal cerebral ischemia were incubated with a κB-oligonucleotide. Although extracts (20 μg) from spleens (S) of p50 knockout mice still showed considerable binding, no bands were detectable in brain extracts from the ischemic (L) or nonischemic side (R). wt, 5 μg of protein extract from wild-type spleen (S).

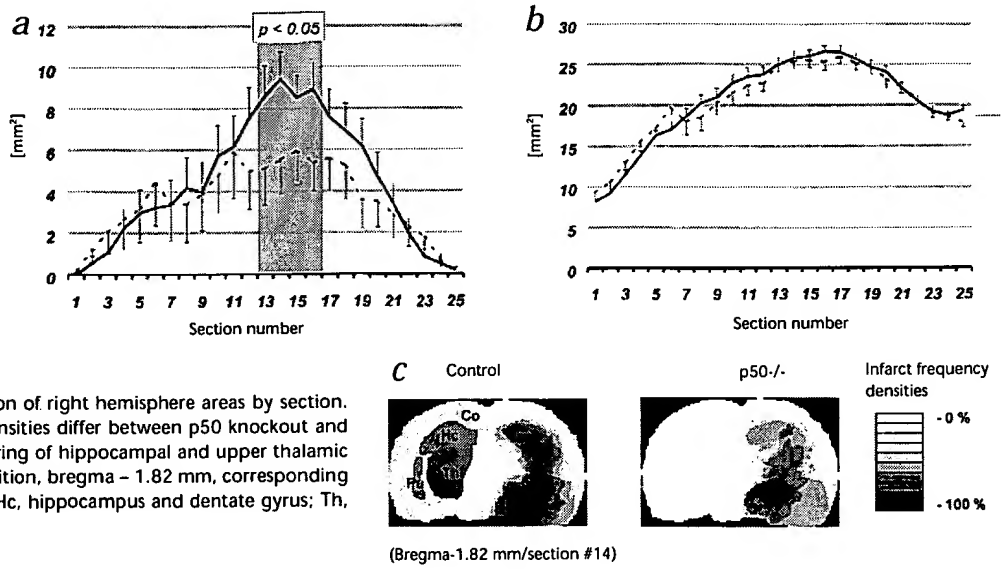

Fig. 5 Ischemic damage is reduced in mice deficient in the p50 subunit of NF-κB. ***a***, Corrected infarct areas in consecutive brain sections. There were significant differences in regional infarct volume (sections 13–16): $P < 0.05$, p50 knockout mice (dashed line) compared with control (solid line) mice (2 h of media occlusion and 20 h of reperfusion). ***b***, Brain volumes in p50 knockout mice (dashed line) and wild-type (solid line) mice are identical, as demonstrated by a comparison of right hemisphere areas by section. ***c***, Regional infarct frequency densities differ between p50 knockout and control mice with a relative sparing of hippocampal and upper thalamic areas in p50 knockout mice (position, bregma – 1.82 mm, corresponding to section 14 in *a*). Co, cortex; Hc, hippocampus and dentate gyrus; Th, thalamus; Pu, putamen.

been seen[22]. The mechanism of the long-lasting activation after ischemia has not been determined, but could be due to stimuli constantly present in the brain after ischemia or cell-autonomous positive-feedback loops.

The absence of the p50 subunit of NF-κB resulted in a significant reduction in infarct size in parts of the vascular territory of the middle cerebral artery. Brain regions that were relatively spared, by analysis of infarct frequencies in the coronal plain in p50 knockout mice, included the hippocampus and the upper thalamus (Fig. 5*c*), which constitute part of the ischemic penumbra, the main area at risk for delayed neuronal death. In these regions, NF-κB was activated in cerebral ischemia (Fig. 2). The idea of a cell death-promoting role of NF-κB with stimuli present in focal ischemia is supported by a protective effect of NF-κB 'decoy' oligonucleotides against glutamate-mediated cell death in a human neuronal cell line.

The exact mechanism by which NF-κB contributes to neuronal cell death is unclear at present, and it still remains to be clarified which target genes are dependent on NF-κB activation in our model. We could not detect consistent co-localization of activated NF-κB to TUNEL-positive neurons after various reperfusion times (data not shown), which might be explained by the fact that NF-κB is no longer detectable when DNA degradation begins in the process of neuronal death after focal ischemia. However, we did observe partial co-expression of activated NF-κB with the FAS-ligand, an NF-κB target gene[23], and with its receptor (FAS/CD95), which are prescriptive cell death markers in neurons[24].

So far, NF-κB has resisted attempts to generalize its function in cell death in several well-studied systems, such as cancer or the immune system[5]. Our data indicate a cell death-promoting role of NF-κB in focal cerebral ischemia. Evidence for NF-κB activation has also been presented in other important examples of neurodegeneration, such as Alzheimer disease[25,26] and Parkinson disease[27]. It can be activated by kainate-induced seizures[10] and was inducible through the p75 low-affinity NGF receptor that is involved in neuronal cell death[28]. Induction has also been shown in a model of global ischemia[22,29].

Although a pro-apoptotic role of NF-κB in ischemia or glutamate-related damage is supported by others[30,31], there is emerging data from cell culture models that NF-κB may counteract cell death in neurons induced by β-amyloid[32,33]. A dual action of NF-κB in global ischemia, whereby it may be either protective or detrimental depending on the onset and duration of its activation, has been proposed[29]. Therefore, NF-κB may in fact prove to be 'Janus-faced' in the nervous system for cell death, depending on the circumstances in which it is activated[34].

Although activation of NF-κB is an early step in the pathogenesis of cerebral ischemia (Fig. 4), its long-lasting action makes it a possible target for pharmacological intervention in stroke. Aspirin, one of the rare agents with proven benefit in acute stroke, is a known inhibitor of NF-κB (ref. 35). Moreover, a reduction in excitotoxicity-induced cell death in neuronal cultures by aspirin correlates with decreased NF-κB activity[30]. Other inhibitors of NF-κB activation are available[36,37] and await testing in cerebral ischemia. In models of other diseases, such as oxidative liver injury after transplantation[38], myocardial infarction[39] or colitis[40], gene therapy approaches have been successfully applied to limit NF-κB-mediated damage. These strategies may also be applicable to focal ischemia.

Methods

Ischemic model. For middle cerebral artery occlusion and reperfusion, the filament model was used. A 5-0 nylon suture (Ethilon, Norderstedt, Germany) with a blunted tip was inserted into the external carotid artery and advanced into the internal carotid until the laser doppler signal decreased considerably. For anesthesia, halothane (1%) and N_2O/O_2 (70%/30%) were used. Mice were kept at a body temperature of 37 °C on a heating pad. Mice were re-anesthetized and the filament was removed after 2 h of ischemia. To obtain physiological parameters, the right femoral artery was cannulated in mice completely sedated by ongoing halothane anesthesia, with blood pressure being continually recorded and samples for blood gas analysis taken 15 min before middle cerebral artery occlusion and 1 h after middle cerebral artery occlusion began. For laser doppler measurements, the probe (P403; Perimed, Järfälla, Sweden) was placed 3 mm lateral and 6 mm posterior to the bregma. Relative perfusion units were obtained (Periflux 4001; Perimed, Järfälla, Sweden).

Assessment of infarct volume. 129/SV X C57BL/6 outbred mice (strain 100 903; Jackson Laboratories, Bar Harbor, Maine) were used as control

mice; this strain provides adequate controls for the mixed background of the p50 knockout mice (http://www.jax.org), circumventing known differences in infarct susceptibility between different mouse strains[41,42]. The p50 knockout mice were provided by D. Baltimore. After 20 h of reperfusion, mice were deeply anesthetized (Rompun™/Ketanest™) and perfused intracardially with saline. Brains were immediately frozen on dry ice. Coronal cryosections (20 μm in thickness) were cut every 400 μm, starting rostrally. Sections were stained with a silver staining technique, which was shown to be equivalent to other common staining techniques for detecting infarcted tissue[43]. Stained sections were directly scanned at 600 dpi and the infarct area was measured (NIH Image 1.61). The total infarct volume was obtained from integrating infarcted areas and correcting for brain edema[44]. Mice without infarct were excluded from the analysis, as this correlated with a low laser doppler drop during ischemia (more than 25% of perfusion retained) and was probably due to an inadequate position of the nylon filament. All data are given as mean ± standard error of the mean (s.e.m.). Values were considered significant at $P < 0.05$ (Student's *t*-test). To generate infarct frequency distribution maps[18], the respective sections of each series were scanned, and infarcts were delineated and projected onto an outline of the respective section. Averaging was done with NIH Image 1.61.

RT-PCR. RNA was isolated in parallel from both cerebral hemispheres of κB5 mice[14]. Infarct was verified by 2,3,5-Triphenyltetrazolium Chloride (TTC) staining of a section 1 mm in thickness[41]. Reverse transcription used 10 μg total RNA using MMLV reverse transcriptase (Promega) and random hexamer primers. The efficiency of the reaction was monitored by trace-labeling the reaction products with ^{32}P-dCTP. Equal amounts of cDNA (20 ng) were used in a set of PCR reactions with a set of primers to amplify β-actin (as described[14]) and cyclophilin (5'-primer: 5'-ACCCCACCGT-GTTCTTCGAC-3'; 3'-primer: 5'-CATTTGCCATGGACAAGATG-3') as internal standards and a set of primers to amplify the κB5-transgene-specific transcript[14]. Products were separated by size on a 5% polyacrylamide gel and quantified using a phosphorimager (Fuji BAS 1000). The phosphorimager units measured were plotted onto a logarithmic graph, and areas within the linear range of the graph were used to compare the two hemispheres, after internal standards showed equal amplification patterns[15].

Electrophoretic mobility shift assay. Brain extracts were prepared by grinding the tissue on dry ice and adding two volumes of buffer C (ref. 45), followed by three 'freeze–thaw' cycles and centrifugation at 13,000*g* for 5 min. Protein (20 μg unless otherwise indicated) and a double-stranded oligonucleotide from the κB-enhancer (5'-GATCCAGAGGGGACTTTCC-GAGA-3') labeled with ^{32}P by a Klenow fill-in reaction were incubated at room temperature in the following buffer: 1 mM EDTA, 0.5 μg/μl BSA, 10 mM TrisCl pH 7.5, 50 mM NaCl, 1 mM DTT, 5 % glycerol and 0.15 μg/μl polydIdC (total volume, 20 μl). For competition experiments a mutated consensus binding sequence (5'-GATCCAGACCATGGTATCCGAGA-3') was added. Protein–DNA complexes were resolved on a 6% nondenaturing polyacrylamide gel at 280 V. Antisera for supershift experiments have been described[46].

Immunohistochemistry. For the evaluation of activation patterns, cryosections 40 μm in thickness from ischemic rat brain were fixed in 4 % paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100 overnight, and incubated with a monoclonal RelA antibody (Boehringer). A biotinylated secondary antibody (donkey-anti-mouse; Dianova, Hamburg, Germany) and the ABC kit (Vector, Burlingame, California) were used for detection, following the manufacturer's instructions. TUNEL labeling for immunofluorescence was done by incubation with 0.5 U/μl terminal transferase (Boehringer) and 6 pmol/μl fluorescein-coupled dUTP (Amersham) for 1 h in a humidified chamber. For double labeling, polyclonal antibodies against GFAP (Boehringer), NeuN (Chemicon, Temecula, California) and neurofilament-200 kD (Sigma) were used and detected with a Cy3- or fluorescein-marked secondary antibody (Dianova, Hamburg, Germany).

Cell culture 'decoy' experiment. NT2 precursor cells (Stratagene, La Jolla, California) were cultured and differentiated to hNT neurons according to the manufacturer's recommendations. The hNT neurons were provided by R. Brandt. For the 'decoy' experiment, differentiated neurons were plated in collagen-coated 24-well plates (Falcon, Heidelberg, Germany) at a density of 1 × 10^5 neurons/well. Cells were maintained in DMEM/F12 medium (Sigma), containing 15 % FCS (Life Technologies). At 2 h before the glutamate pulse, 12.5 μM double-stranded phosphorothioate oligonucleotides were added, containing either the wild-type or the mutated NF-κB consensus binding motif used in the gel-shift experiments. Then, 1 mM glutamate was applied for 30 min in the presence of the 'decoy' oligonucleotides in a buffer containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 15 mM glucose and 25 mM HEPES, pH 7.4. Then, cells were washed with PBS and maintained in DMEM/F12 medium containing 15 % FCS and 'decoy' for 24 h. After lysis, the amount of DNA–histone complexes, as an indicator of cell death, was measured by ELISA (cell-death-detection ELISA-plus; Boehringer), following the manufacturer's instructions.

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